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Aberrant insulin signaling results in an impaired ability to clear glucose from the bloodstream, which progresses to type II diabetes mellitus once insulin stimulation no longer results in a significant decrease in blood glucose levels. GLUT4 is the insulin-responsive glucose transporter which, upon insulin stimulation, translocates to the plasma membrane where it is embedded, promoting glucose uptake in muscle and adipose tissue. The potential causes for insufficient glucose uptake are numerous because the insulin signaling pathway has many intermediates and targets, including myosin II. It is known that myosin II is necessary for proper glucose uptake, though the mechanism by which myosin II promotes glucose uptake is unknown. We aim to determine if myosin II is functioning to stabilize the actin tether which is used as a track by secretory GLUT4 vesicles to reach the plasma membrane. GLUT4 vesicle trafficking along the actin tether is accomplished via association of the GLUT4 vesicle with the cargo carrying, actin-based motor proteins, myosin Va and myosin Ic. Here we demonstrate that myosin II is necessary to localize myosin Va and myosin Ic with GLUT4 and F-actin at the plasma membrane. These findings demonstrate that myosin II is necessary to localize these proteins in an insulin-stimulated acto-myosin protein complex which supports GLUT4 translocation to the plasma membrane. These events may be critical regulatory nodes which could yield therapeutic targets helpful in treating those with type II diabetes.

THE ROLE OF MYOSIN II IN STABILIZING ACTIN TETHERS PROMOTING GLUT4 EXOCYTOSIS IN 3T3-L1 ADIPOCYTES

by

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Master of Science

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Approved by

_____
Committee Chair

Dedicated to my mother, Jeannie.

♥

APPROVAL PAGE

This thesis written by Jonah B. Nikouyeh has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Insulin-stimulated glucose uptake in adipose tissue and skeletal muscle is important for whole-body glucose homeostasis. Diabetes mellitus, characterized by increased blood glucose levels, is a disease affecting approximately 10% of Americans[1]. There are two types of diabetes mellitus, type I and type II. Type I diabetes is an autoimmune disorder wherein the β cells of the pancreas, the cells which synthesize insulin, are destroyed by the body's immune system. Thus, no insulin-stimulated glucose uptake can occur without exogenous insulin administration. Type II diabetes differs from type 1 diabetes in that insulin is produced, however, the tissues which are insulin-responsive, adipose and muscle tissue, become unresponsive to insulin stimulation. This is brought about by deficiencies in insulin signaling. In adipocytes and myocytes, insulin binds to its receptor activating several intracellular pathways, most notably the PI3K/AKT pathway. This pathway facilitates the translocation of vesicles containing the insulin responsive glucose transporter, GLUT4, to the plasma membrane where it is embedded and promotes glucose uptake[2] (Figure 1).

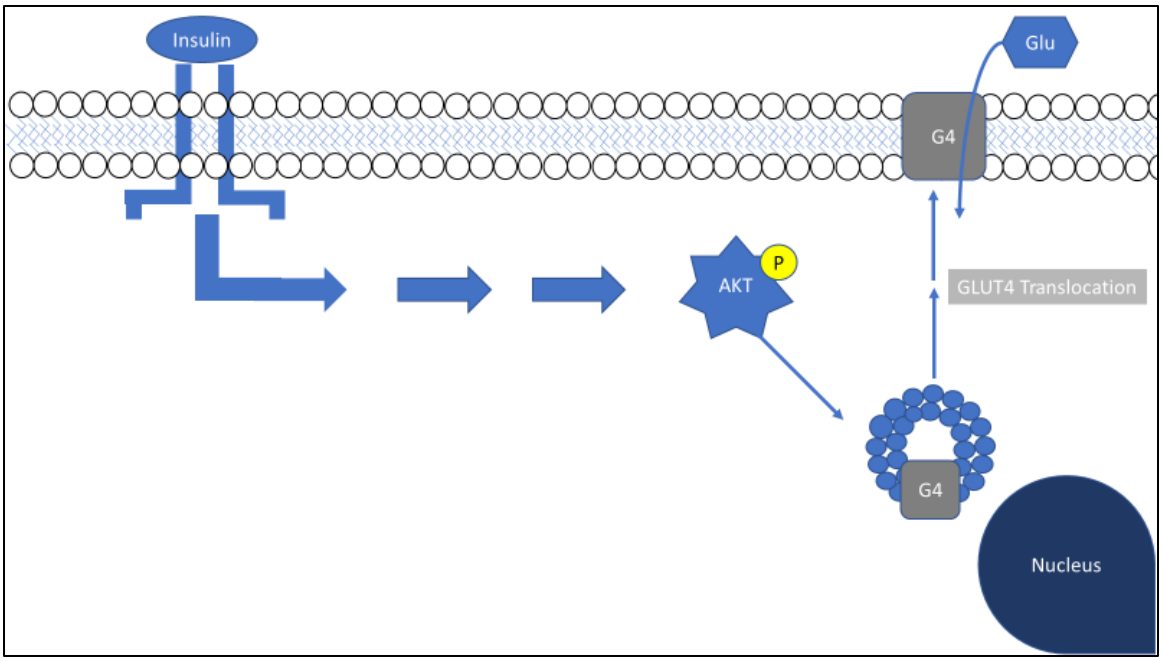


Figure 1. Schematic of Insulin-stimulated Glucose Uptake via Activation of the PI3K/AKT Signaling Pathway.

Before GLUT4 vesicle fusion with the plasma membrane can occur, the actin cytoskeleton must undergo remodeling. In adipocytes, few stress fibers are present thus the vast majority of filamentous actin, F-actin, resides directly underneath the plasma membrane at the cell cortex. By virtue of its location within adipocytes, actin acts as an important regulator of vesicle exocytosis in two ways. First, extensions of cortical actin, known as actin tethers, reach into the cytosol and have been implicated in acting as tracks for secretory cargo to reach the plasma membrane after reaching the end of microtubules[3]. Second, F-actin must be reorganized in order to allow for vesicle exocytosis as the actin cytoskeleton acts as a physical barrier preventing membranes of secretory vesicles from fusing with the plasma membrane[4].

Myosin II is an important protein regulating the actin cytoskeleton as it binds cortical actin and provides the necessary contractile force to drive various cytoskeletal-dependent functions such as cytokinesis, cellular migration, and the maintenance of cortical tension[5,6]. The role of myosin II in recent years has expanded to include mediating vesicle exocytosis as exemplified in the myosin II-dependent release of catecholamines from murine chromaffin cells[7]. We have shown previously that GLUT4 mediated glucose uptake is reduced in the presence of the myosin II specific inhibitor, blebbistatin[8]. These findings further suggest that myosin II is necessary for vesicle exocytosis. What is not known is the molecular mechanism(s) by which myosin II mediates GLUT4 vesicle exocytosis.

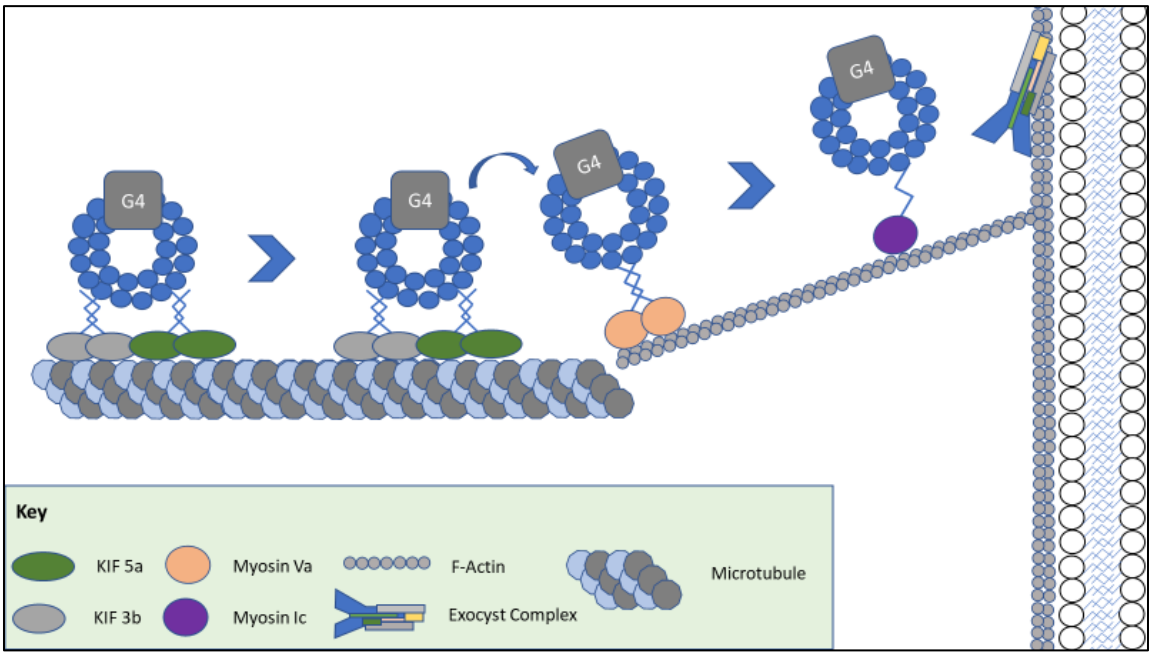


Figure 2. Schematic of Translocation of GSVs from Microtubules to the Actin Tether.

GLUT4 Trafficking

GLUT4 trafficking is a highly regulated, multi-step process[3,9]. In the basal (unstimulated) state, GLUT4 is present in the cytoplasm embedded in the membrane of intracellular GLUT4 storage vesicles (GSVs) in a perinuclear region. Upon insulin stimulation, GSVs translocate to the plasma membrane on microtubules via microtubule-associated kinesin motor proteins, KIF3b and KIF5a[10,11]. Upon approaching the plasma membrane, GSVs disassociate from the microtubule and translocate to actin tethers extending approximately 200 nm into the cytoplasm[9]. Two myosin motor proteins, myosin Va and myosin Ic, have been implicated in regulating GSV translocation to the cell cortex along actin tethers[12–15] (Figure 2).

In the basal state, myosin Va is diffusely localized throughout the cytosol; however, with insulin stimulation myosin Va translocates and colocalizes with GLUT4 at or near the plasma membrane. Complimentary immunoprecpitation studies revealed a physical association of myosin Va with GSVs. Additionally, anti-myosin Va siRNA inhibited GLUT4 translocation and reduced glucose uptake, further suggesting myosin Va is responsible for promoting anterograde GSV translocation and ultimately GLUT4-dependent glucose uptake[12].

Like myosin Va, myosin Ic shows distinct localization patterns within 3T3-L1 adipocytes[13,16]. In the basal state, myosin Ic shows a punctate patterning in the cytoplasm. With insulin stimulation, myosin Ic has also been shown to colocalize with GLUT4 and bind F-actin at the plasma membrane. Additionally, myosin Ic associates with the exocyst, a “y”-shaped, octomeric protein complex responsible for tethering secretory

vesicles to the plasma membrane[14,17]. This suggests a role for myosin Ic in targeting GSVs to the exocyst complex for exocytosis. Inhibition of myosin Ic via expression of a myosin Ic dominant inhibitory cargo domain results in a marked decrease of GLUT4 embedded in the plasma membrane. Furthermore, the role for myosin Ic in vesicle exocytosis has been established in other cell lines, specifically alveolar type II cells which demonstrate that a knockdown of myosin Ic expression decreases surfactant vesicle exocytosis[18].

Once the GSV arrives at the cell cortex via myosin Va and myosin Ic, proteins embedded in the GSV form a complex with plasma membrane bound proteins forming what is known as the SNARE complex. The SNARE complex brings both membranes together to mediate a zipper-like fusion of the GSV membrane and the plasma membrane[19]. This results in GLUT4 insertion in the plasma membrane.

Myosin II

Alongside myosin Ic and Va, the conventional myosin, myosin II, has been implicated in promoting GLUT4 vesicle trafficking[8,20,21]. Myosin II is a hexameric protein consisting of two heavy chains which comprise the tail domain, two regulatory light chains (RLC), and two essential light chains (ELC), together comprising the neck domain[22] (Figure 3). Myosin II also has an ATPase head domain that interacts directly with actin to mediate actin binding[23].

Two forms of myosin II are expressed within mammals: muscle and non-muscle myosin II. The more commonly known myosin is muscle myosin II which assembles into

bipolar filaments via interactions of many myosin II tail domains. Head domains present at each end of the bipolar filaments mediate actin contraction and relaxation in sarcomeres, causing contraction and relaxation of entire muscle fibers. In a non-muscle cell context, non-muscle myosin II shares the same structure and ability to assemble into bipolar filaments as muscle myosin II; however, non-muscle myosin II participates in a variety of critical actin cytoskeletal processes ranging from cytokinesis to cell migration[5,6]. Three isoforms of non-muscle myosin II are encoded within mammalian genomes, non-muscle myosin II A, B, and C; however, within 3T3-L1 adipocytes only the A and B isoforms (referred to hence as myosin IIa and myosin IIb) are expressed[8,24,25]. Within adipocytes, the myosin II isoforms show distinct localization patterns. Myosin IIa, under basal conditions, resides at the perinuclear region, much like GLUT4. When stimulated by insulin, myosin IIa translocates to the plasma membrane. In contrast, myosin IIb is enriched at the cell cortex under basal conditions and remains at the cell cortex with insulin stimulation[8].

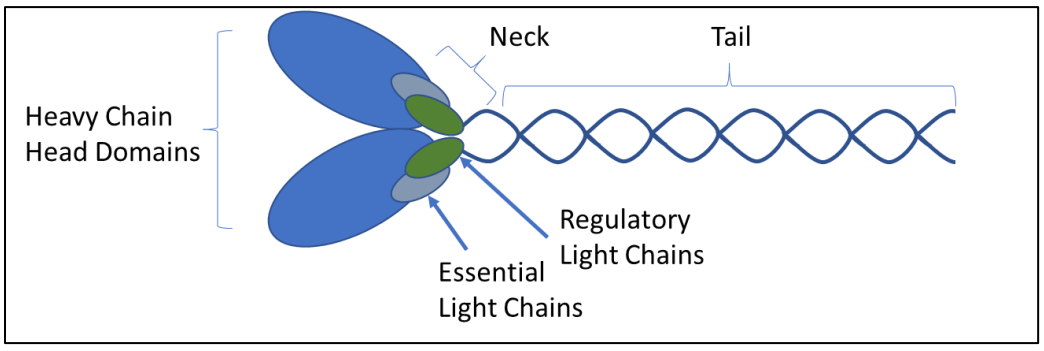


Figure 3. Structure and Protein Domains of Myosin II.

The Regulatory Role of Myosin IIa in GLUT4 Exocytosis

Myosin II is necessary for GLUT4 membrane trafficking during insulin stimulation. We have shown in previous studies that the myosin IIa isoform is regulated by insulin stimulation. Upon insulin stimulation, the RLC of myosin IIa is phosphorylated by myosin light chain kinase (MLCK) which promotes the translocation of myosin IIa from the perinuclear region to the plasma membrane. However, the RLC of myosin IIb remains unchanged during insulin stimulation. Myosin IIb resides at the cell cortex in the basal state and remains at the cell cortex upon insulin stimulation. RLC phosphorylation via MLCK also increases myosin II ATPase activity by allowing myosin II to linearize from its inactive, bipolar filament assembly incompetent 10S hairpin conformation[26,27]. Prior to myosin IIa translocation, GLUT4 is also stimulated to translocate from the perinuclear region to the plasma membrane[28]. In order for appropriate glucose uptake to take place, both GLUT4 and myosin IIa must be recruited to the plasma membrane[20].

In a previous study, we treated adipocytes with blebbistatin, a reversible chemical inhibitor that inhibits myosin II ATPase activity[20,29]. Blebbistatin has been shown to be a specific myosin II inhibitor with no cross reactivity with myosins I, V, or X[30]. Treatment with blebbistatin prevented adequate glucose uptake despite GLUT4 translocation to the plasma membrane which appeared to be a consequence of a decrease in GLUT4 insertion in the plasma membrane. This was demonstrated by staining adipocytes with an antibody against an exofacial domain of GLUT4[28]. Thus, myosin II is critical for the insulin-stimulated insertion of GLUT4 in the plasma membrane.

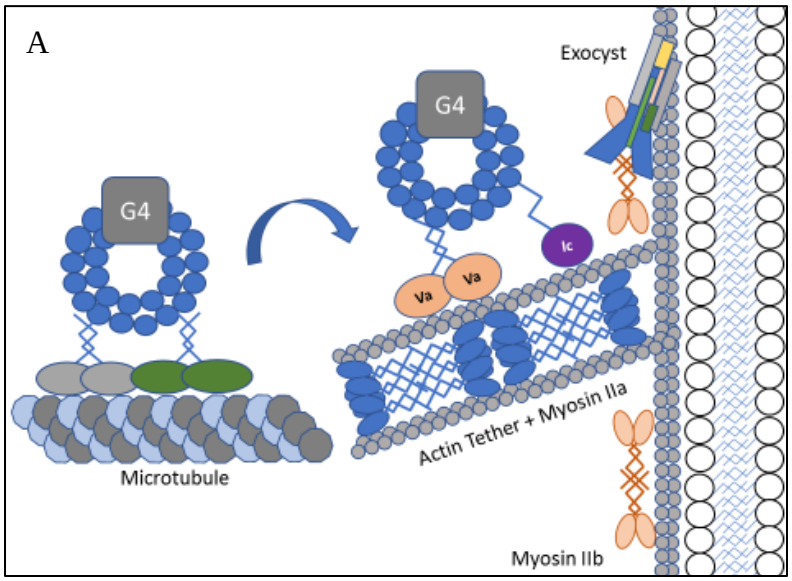
Cortical Actin and Actin Tethers

Though myosin II appears to be vital for proper GLUT4 exocytosis, myosin II cannot exert contractile forces on the cytoskeleton necessary for GLUT4 exocytosis without binding to actin. Within adipocytes, there exists an enrichment of cortical F-actin. It has been shown that with insulin stimulation, F-actin at the plasma membrane increases suggesting a role for actin filaments in GLUT4 translocation[20]. To investigate this potential role, Kanzaki et. al exposed adipocytes to compounds which either prevent actin depolymerization or inhibit actin polymerization, enhancing cortical actin and reducing cortical actin, respectively[4]. Both states of the cortical actin network prevented adequate glucose uptake making a case for the necessity of a dynamic actin cytoskeleton which can polymerize in specific areas to act as a scaffold for proteins which increase GLUT4 exocytosis efficiency. Actin tethers are an example of such scaffolds.

Actin tethers are extensions of cortical actin which can bridge distances within 100 to 200 nm of the plasma membrane. This is much greater than the effective tethering distance of the exocyst complex which is approximately 15 nm[3]. These tethers reach into the cytosol towards microtubules which carry GSVs towards the cell periphery. As described earlier, it is at this point that the microtubule-bound kinesin proteins carrying GSVs "hand off" the GSVs to the actin-based motor proteins myosin Va and Ic for advancement to the cell cortex along the actin tether. Therefore, it is necessary to have a rich, dynamic acto-myosin cytoskeleton to promote GLUT4 exocytosis.

This was further demonstrated by Stall et al where it was shown that with insulin stimulation there is an enhanced co-localization of F-actin and myosin IIa at the plasma

membrane. With insulin stimulation, an increase in F-actin at the plasma membrane was also observed. When treated with blebbistatin, insulin-induced F-actin enrichment and co-localization with myosin IIa at the plasma membrane decreased[20]. This suggests a role for myosin IIa in stabilizing F-actin which accumulates at the plasma membrane under insulin-stimulated conditions. Indeed, in *Dictyostelium* it has been shown that myosin II acts to cross link actin filaments, a process necessary to maintain cortical tension and proper cell morphology. *Dictyostelium* lacking myosin II are more susceptible to extreme cell body deformation, including fragmentation, when chemotaxing through a restrictive environment[31–33].



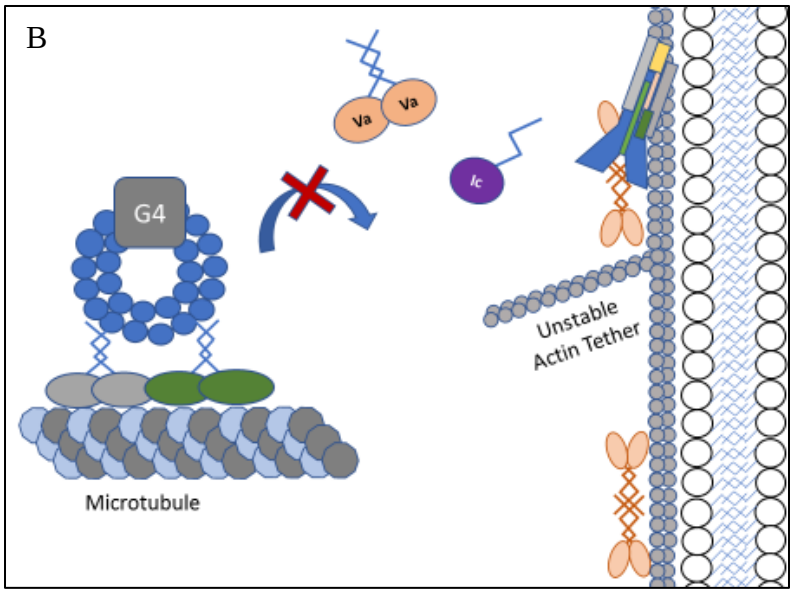


Figure 4. Proposed Mechanism of Myosin II Stabilization of Actin Tethers. A) Proposed model of myosin II stabilization of actin tethers at the plasma membrane promoting GLUT4 exocytosis. **B)** Proposed inhibition of myosin II prevents the formation of stable actin tethers, inhibiting GLUT4 exocytosis.

These insights into the rich cortical actin network of adipocytes, the role of actin tethers as "tracks" for anterograde vesicle movement, and the necessity for myosin IIa in GLUT4 exocytosis demonstrate a case supporting myosin IIa and F-actin tethers as critical regulators of GLUT4 exocytosis. However, what remains unknown is the mechanism by which myosin II is able to promote GLUT4 exocytosis. More specifically, it is unknown in which vesicle trafficking events, such as tethering or docking and fusion, myosin II is participating. Given the available evidence, a model emerges in which myosin II functions to stabilize actin tethers at the plasma membrane (Figure 4 A/B). Thus, adipocytes stimulated by insulin have increasing levels of F-actin at the plasma membrane. Upon insulin stimulation, myosin IIa and GLUT4 are independently stimulated to translocate to the plasma membrane. Myosin IIa stabilizes actin filaments

at the plasma membrane, more specifically actin tethers, which allows cargo-binding, actin-based motor proteins such as myosin Va and Ic to facilitate GSV procession towards the plasma membrane. In the present study, we investigate the role of myosin II in stabilizing actin tethers in 3T3-L1 adipocytes, promoting GLUT4 exocytosis.

CHAPTER II

MATERIALS AND METHODS

Materials

3T3-L1 pre-adipocytes (CL-173) were purchased from ATCC. Dulbecco's Modified Eagle Medium (11995) and fetal bovine serum (26140-079) were purchased from Gibco. Bovine calf serum was purchased from Hyclone (SH20072.03). Insulin (15500-1G) and 3-isobutyl 1-methylxanthine (I5879) were obtained from Sigma. Antibodies for GLUT4 (sc-1606), Myosin Va (sc-365986), Myosin Ic (sc-136544), and HRP conjugated donkey anti-goat secondary antibody (sc-2020) were purchased from Santa Cruz Biotechnology. Agarose Beads conjugated with Protein A+G (sc-2003) was also purchased from Santa Cruz Biotechnology. Actin antibody was purchased from Abcam (ab3280). Myosin IIa (M8064) and Myosin IIb (M7939) antibodies were obtained from Sigma. Alexa-flour 488 conjugated rabbit anti-mouse antibody (A-11059), Alexa-fluor 594 conjugated donkey anti-mouse antibody (A-11058), Alexa-fluor 488 donkey anti-rabbit antibody (A-21206), Alexa-fluor 594 goat anti-rabbit antibody (R37117), and Texas Red-X Phalloidin (T7571) was obtained from Invitrogen. Blebbistatin (Ab120425) was purchased from Abcam. The enhanced chemiluminescence (ECL) kit (K-12045-D20) was obtained from Advansta.

Cell Culture

3T3-L1 preadipocytes were cultured in Dulbeccos's Modified Eagle Medium (DMEM) supplemented with 10% calf serum every two days until confluent. Two days after confluency, differentiation was induced by supplementing DMEM with 0.5 mM 3-isobutyl 1-methylxanthine, 1 µM dexamethasone, 1 µg/ml of insulin and 10% fetal bovine serum (FBS). On day 3 after differentiation, media was replaced with 10% FBS + DMEM supplemented with 1 µg/ml of insulin. On day 5 and every two days after, the media was replaced with 10% FBS + DMEM. Between days 8-10, the cells became fully differentiated and were used for experiments.

Immunostaining

Day 8-10 differentiated 3T3-L1 adipocytes grown on cover slips were serum starved in the presence of 100 uM blebbistatin or vehicle (0.1% DMSO) for 4 hours and were left untreated (Basal) or stimulated with 100 nM insulin in the absence of blebbistatin (Insulin) or in the presence of blebbistatin (Insulin + Blebbistatin) for 30 min. Cells were then fixed with 3.7% paraformaldehyde, permeabilized with 0.25% Triton X-100 for 5 minutes, and then blocked in a 10% donkey serum - 1x PBS solution. Coverslips were incubated with anti-GLUT4 antibody or phalloidin conjugated with Texas Red, and either, anti-myosin Va antibody, or anti-myosin 1c antibody. Coverslips were subsequently incubated with the appropriate fluorescently labeled secondary antibodies. Coverslips were mounted on slides using Dako fluorescent mounting medium (S3023). Slides were viewed using fluorescence microscopy using a Keyence BZ-X710

Fluorescent Microscope. Additional images were also acquired using confocal microscopy via an Olympus IX81 Motorized Inverted Confocal Microscope and FLUOVIEW FV500 software.

Co-immunoprecipitation

Once adipocytes were serum starved and treated as described in the immuno-staining section, cells were washed twice with ice-cold 1x PBS and then exposed to lysis buffer containing 100 mM NaF, 1 M HEPES, 5 M NaCl, 100 mM EDTA, 70% NP-40, 100 mM NaPPi, 50% Glycerol, protease inhibitor, and phosphatase inhibitor. Protein lysates were collected, rocked for 20 min at 4 ^{o}C, then centrifuged at 6000x g for 20 minutes at 4 ^{o}C to pellet insoluble materials. Protein was then incubated with either anti-myosin IIa antibody or anti-myosin Va antibody overnight at 4 ^{o}C. Subsequent incubation of protein extract with agarose beads conjugated with protein A/G was performed overnight at 4 ^{o}C. Samples were then centrifuged for 5 min at 2500x g at 4 ^{o}C followed by 3 washes with lysis buffer before being stored at -20 ^{o}C for subsequent western blot analysis.

Western Blot

Proteins were extracted from day 8-10 differentiated 3T3-L1 adipocytes as described in the immunoprecipitation section. Extracts were heated to 95 ^{o}C for 5 minutes and subjected to 10% SDS-PAGE and then transferred for 24 hours to a Immobilon-P membrane (Millipore IPFL 00010). Proteins were then probed using specific primary and

secondary antibodies. Protein bands were visualized using enhanced chemiluminescence (ECL) and quantified using densitometric analysis in ImageJ software.

Statistical Analysis

Data are expressed as means ± SEM. The significance of differences between means, set at $P<0.05$, was assessed by Student's t-test (Microsoft Excel).

CHAPTER III

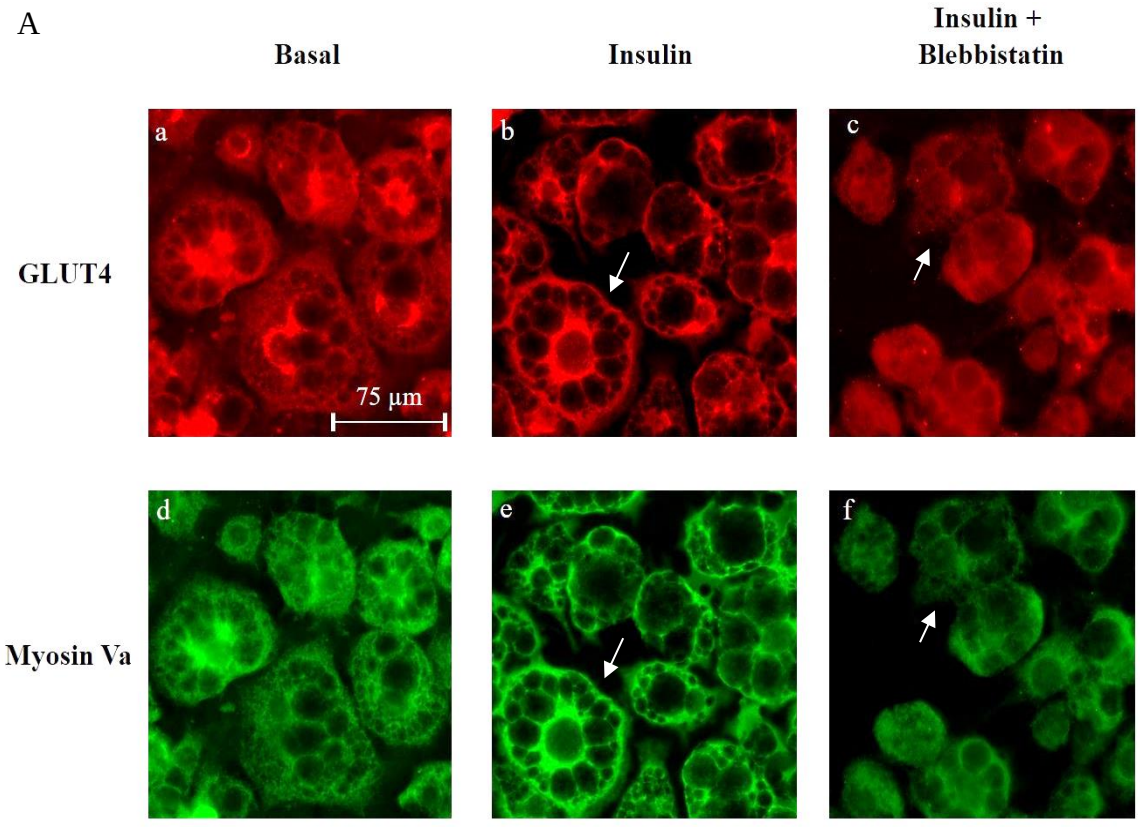
RESULTS

Previous studies in our lab have shown that myosin II inhibition via blebbistatin treatment of adipocytes significantly reduced insulin-stimulated glucose uptake[8]. Previous studies also have shown that myosin II inhibition prevents insulin-stimulated GLUT4 vesicle fusion with the plasma membrane[28]. Together, these results suggest that myosin II is necessary for glucose uptake by facilitating the process of GLUT4 translocation to the plasma membrane, a process which is mediated in part by actin tethers. The focus of these studies is to determine if myosin II is functioning to stabilize the actin tether which is used as a track by secretory GLUT4 vesicles to reach the plasma membrane. GLUT4 vesicle procession along the actin tether is accomplished via association of the GLUT4 vesicle with the cargo carrying, actin-based motor proteins, myosin Va and myosin Ic.

Myosin II Inhibition Reduces Myosin Va Localization with GLUT4 at the Plasma Membrane During Insulin Stimulation

Previous studies have shown that myosin Va is integral to GLUT4 exocytosis and glucose uptake in adipocytes[12,34]. Upon insulin stimulation, myosin Va associates with GLUT4 vesicles. Insulin stimulation also induces myosin Va to translocate to the plasma membrane and bind F-actin. This suggests that myosin Va translocates to the plasma

membrane to facilitate insulin-stimulated glucose uptake by binding and carrying GLUT4 vesicles to the plasma membrane along actin tethers. To investigate if myosin II is stabilizing the actin tether on which myosin Va facilitates GLUT4 translocation, we had to first determine if myosin II was necessary to localize myosin Va with GLUT4 during insulin stimulation. To that end, mature adipocytes grown on coverslips were serum starved for 4 hours with and without blebbistatin and then subsequently stimulated for 30 minutes with 100 nM insulin alone, insulin and 100 uM blebbistatin, or left untreated.



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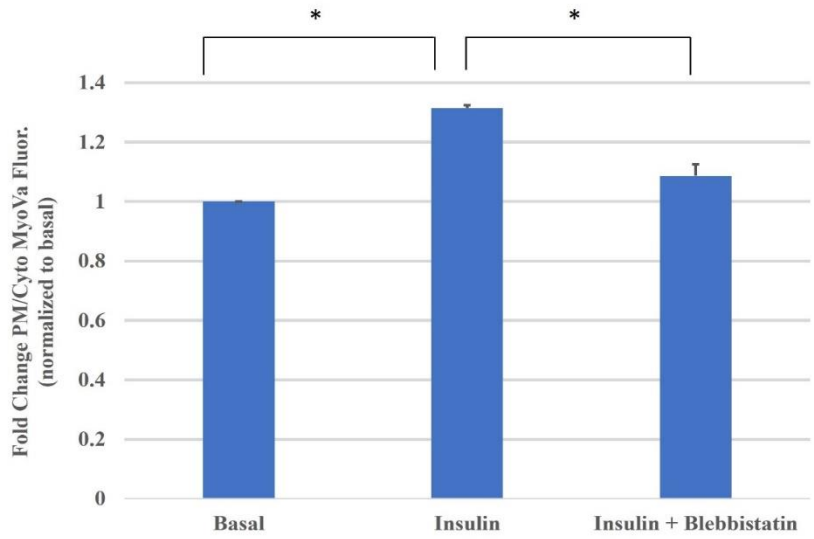

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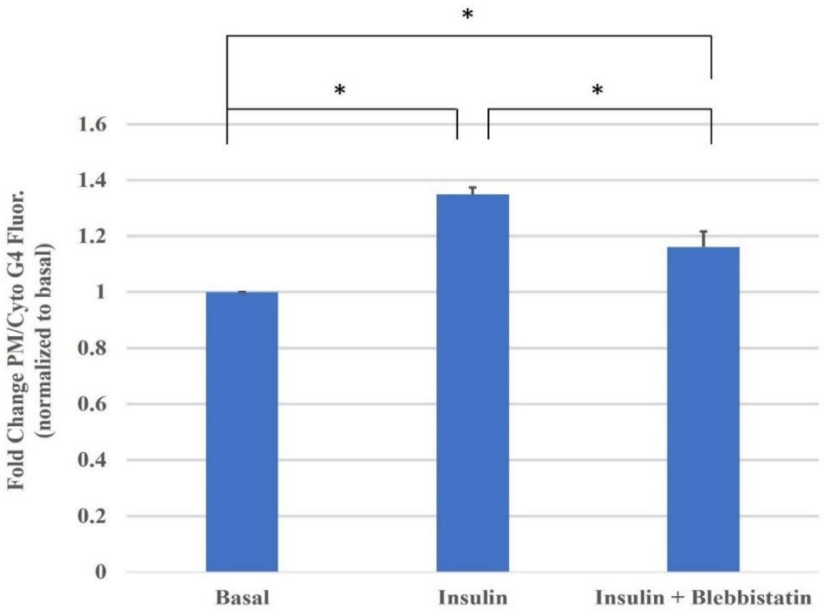
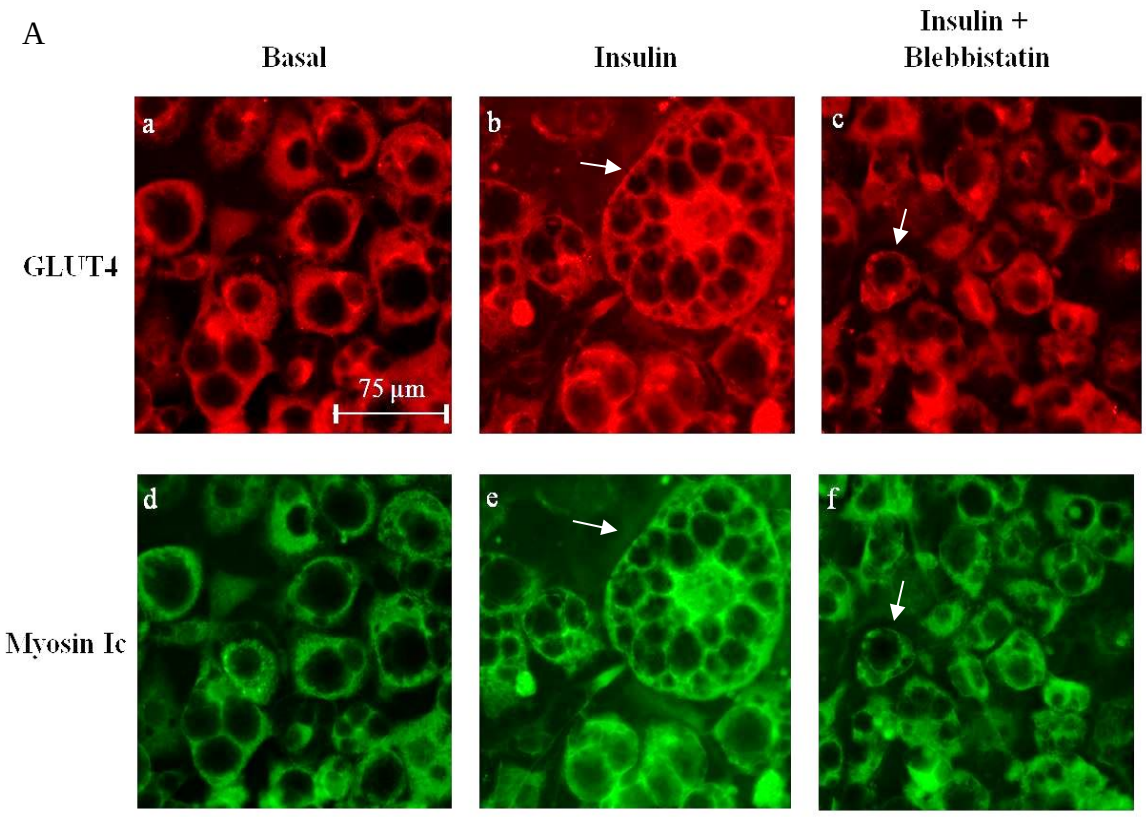


Figure 5. Myosin Va Localization at the Plasma Membrane Decreases with Blebbistatin Treatment. Adipocytes were grown on coverslips and serum starved in the presence of vehicle (0.1% DMSO) or 100 uM Blebbistatin for 4 hours, and then stimulated with or without 100 nM insulin for 30 minutes. Adipocytes on coverslips were fixed, permeabilized, and incubated with antibodies against myosin Va and GLUT4. Appropriate secondary antibodies conjugated to fluorophores were then applied. **A)** Fluorescence was observed using a Keyence BZ-X Fluorescence Microscope. **B-C)** Fold change in fluorescence intensity at the plasma membrane and in the cytosol was quantified using ImageJ and are expressed as percent of vehicle control (basal). Results are means +/- SEM of three independent experiments *$p<0.05$.

Using fluorescence microscopy (Figure 5A) we observed that in the basal state, myosin Va had a cytosolic localization. Upon insulin stimulation, we found that myosin Va redistributes from a mostly cytosolic localization to the plasma membrane. These findings agree with previous studies that characterized myosin Va localization before and during insulin stimulation[12]. It is widely known that GLUT4, upon insulin stimulation, moves from the perinuclear region to the plasma membrane to facilitate glucose uptake. We show that myosin Va and GLUT4 localization at the plasma membrane increased by 31% and 34% respectively when compared to adipocytes in the basal state (Figure 5B and 5C). However, upon myosin II inhibition and insulin treatment, myosin Va and GLUT4 translocation to the plasma membrane only increased by 9% and 16%, respectively. This is a significant reduction in myosin Va translocation to the plasma membrane when compared to insulin stimulation alone. These results suggest that myosin II is necessary to localize myosin Va at the plasma membrane as does GLUT4 under insulin stimulated conditions.

Inhibition of Myosin II Prevents Insulin Stimulated Localization of Myosin Ic and GLUT4 at the Plasma Membrane

While myosin Va has been shown to facilitate GLUT4 translocation to the plasma membrane, it has also been shown that myosin Ic is a necessary component of the process of GLUT4 translocation[13,14]. Thus, we investigated whether myosin II inhibition affected the ability of myosin Ic to localize with GLUT4 at the plasma membrane.


A
Basal
Insulin
Insulin +
Blebbistatin
GLUT4
Myosin Ic
a
b
c
d
e
f
75 µm

B

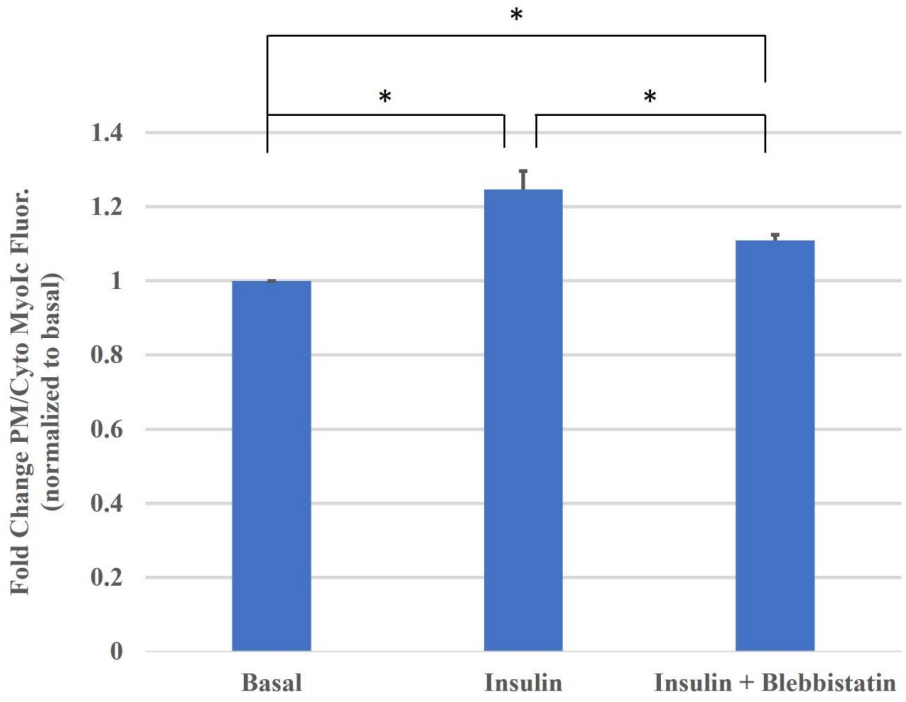


Figure 6. Blebbistatin Inhibition of Myosin II Decreases the Levels of Myosin Ic at the Plasma Membrane During Insulin Stimulation. Adipocytes were grown on coverslips and serum starved in the presence of vehicle (0.1% DMSO) or 100 uM Blebbistatin for 4 hours, and then stimulated with or without 100 nM insulin for 30 minutes. Adipocytes were fixed, permeabilized, and incubated with antibodies against myosin Ic and GLUT4. Appropriate secondary antibodies conjugated to fluorophores were then applied. **A)** Fluorescence was observed using a Keyence BZ-X Fluorescence Microscope. **B)** Fold change in fluorescence intensity at the plasma membrane and in the cytosol was quantified using ImageJ and are expressed as percent of vehicle control. Results are means +/- SEM of three independent experiments. *$p<0.05$

Using fluorescence microscopy (Figure 6A), we have shown that in the basal state myosin Ic is localized to the cytosol. Upon insulin stimulation, myosin Ic signal at the plasma membrane increases, corroborating previous studies which also find an increase in myosin Ic signal at the plasma membrane driven by insulin[13,14]. Myosin Ic signal at the plasma membrane increased by 25% with insulin stimulation (Figure 6B), however, inhibition of myosin II reduced the levels of myosin Ic at the plasma membrane when

compared to insulin stimulation alone. Myosin Ic was primarily detected in a basal localization pattern when treated with blebbistatin and insulin. With insulin and blebbistatin co-treatment, myosin Ic presence at the plasma membrane is only increased 11% from basal, a significant reduction of 14% in myosin Ic translocation when compared to insulin stimulation alone. These findings suggest that, as was seen with myosin Va, myosin II is necessary to facilitate the insulin-dependent localization of myosin Ic with GLUT4 at the plasma membrane.

Myosin II Inhibition Reduces the Association of Myosin Ic and Myosin Va with Myosin II During Insulin Stimulation

Immunoprecipitation studies were employed to determine if the ability of myosin II to complex with either myosin Ic or myosin Va was decreased during myosin II inhibition. It should be noted that blebbistatin is a specific myosin II inhibitor and has been shown to have no cross-reactivity with myosins I or V[30]. Mature adipocytes were serum starved in the presence of 0.1% DMSO or blebbistatin and then treated with insulin or left unstimulated (basal). Whole cell lystates were collected from each treatment condition and incubated with either myosin Va or myosin IIa antibody for immunoprecipitation.

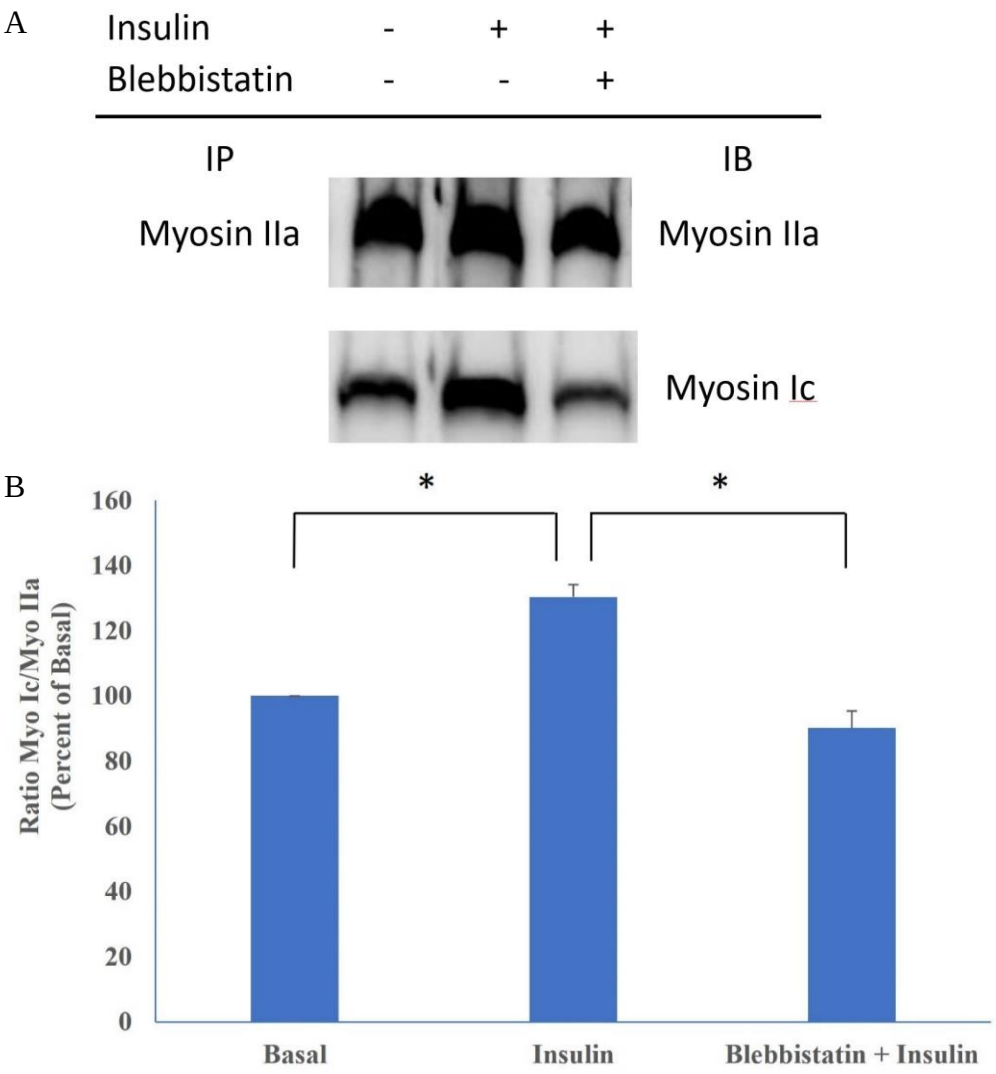
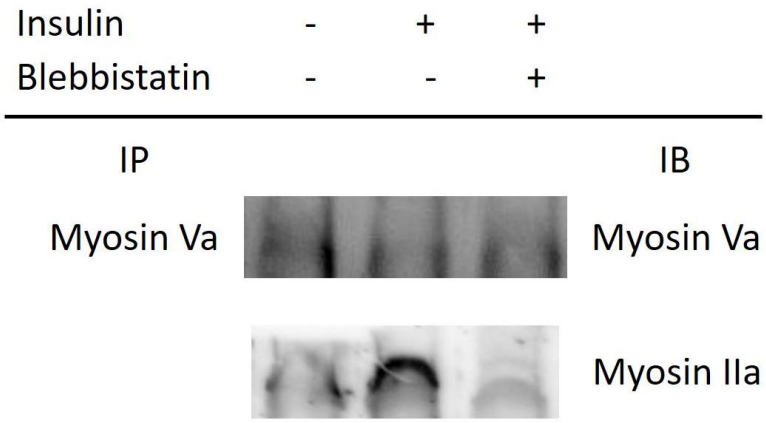


Figure 7. Blebbistatin-mediated Inhibition of Myosin II Decreases the Association of Myosin Ic with Myosin II During Insulin Stimulation. Adipocytes were serum starved in the presence of vehicle (0.1% DMSO) or 100 uM Blebbistatin for 4 hours, and then stimulated with or without 100 nM insulin for 30 minutes. Whole cell lysates were collected and incubated with myosin IIa antibody overnight with agarose beads conjugated with protein A/G. Bound protein was subjected to SDS-PAGE. **A)** Protein was transferred to a membrane and immunoblotted for myosin IIa and myosin Ic. **B)** Fold change in band intensity indicating increased association of protein with myosin IIa was quantified using densiometric analysis in ImageJ and are expressed as percent of vehicle control. Results are means +/- SEM of three independent experiments. *$p<0.05$

It was observed with myosin IIa immunoprecipitation (Figure 7A) that there was a 30% increase in association of myosin Ic with myosin IIa during insulin stimulation when compared to the basal state (Figure 7B). Surprisingly, with blebbistatin and insulin co-treatment, myosin Ic association with myosin IIa decreased by 10% when compared to the basal state (Figure 7B).

A



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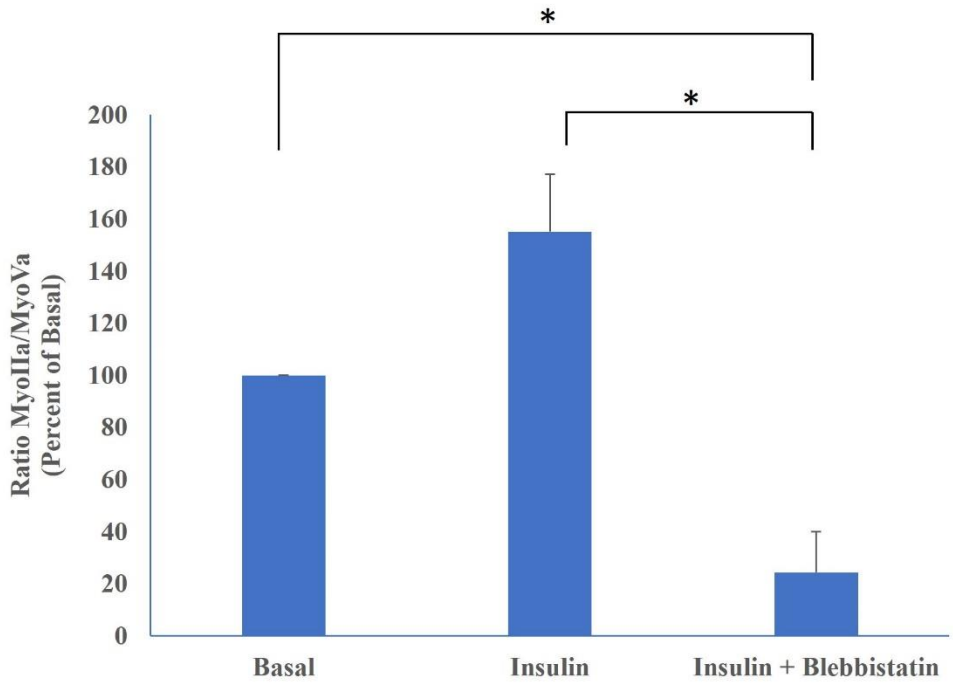


Figure 8. Association of Myosin Va with Myosin II Decreases with Inhibition of Myosin II. Adipocytes were serum starved in the presence of vehicle (0.1% DMSO) or 100 uM Blebbistatin for 4 hours, and then stimulated with or without 100 nM insulin for 30 minutes. Whole cell lysates were collected and incubated with myosin Va Ab overnight with agarose beads conjugated with protein A/G. **A)** Protein was transferred to a membrane and immunoblotted for myosin Va, and myosin IIa, **B)** Fold change in band intensity was quantified using densiometric analysis in ImageJ and are expressed as percent of vehicle control. Results are means +/- SEM of three independent experiments. *$p<0.05$

Myosin Va immunoprecipitation studies (Figure 8 A-B) demonstrated a 55% increase in myosin IIa association with myosin Va during insulin stimulation when compared to the basal state. Blebbistatin and insulin co-treatment reduced myosin IIa association with myosin Va by 75% from basal. A decrease in myosin Va/Ic association with myosin II during myosin II inhibition appears to indicate that myosin II is necessary to maintain the acto-myosin protein complex promoting GLUT4 translocation of which myosin Ic and myosin Va are a part.

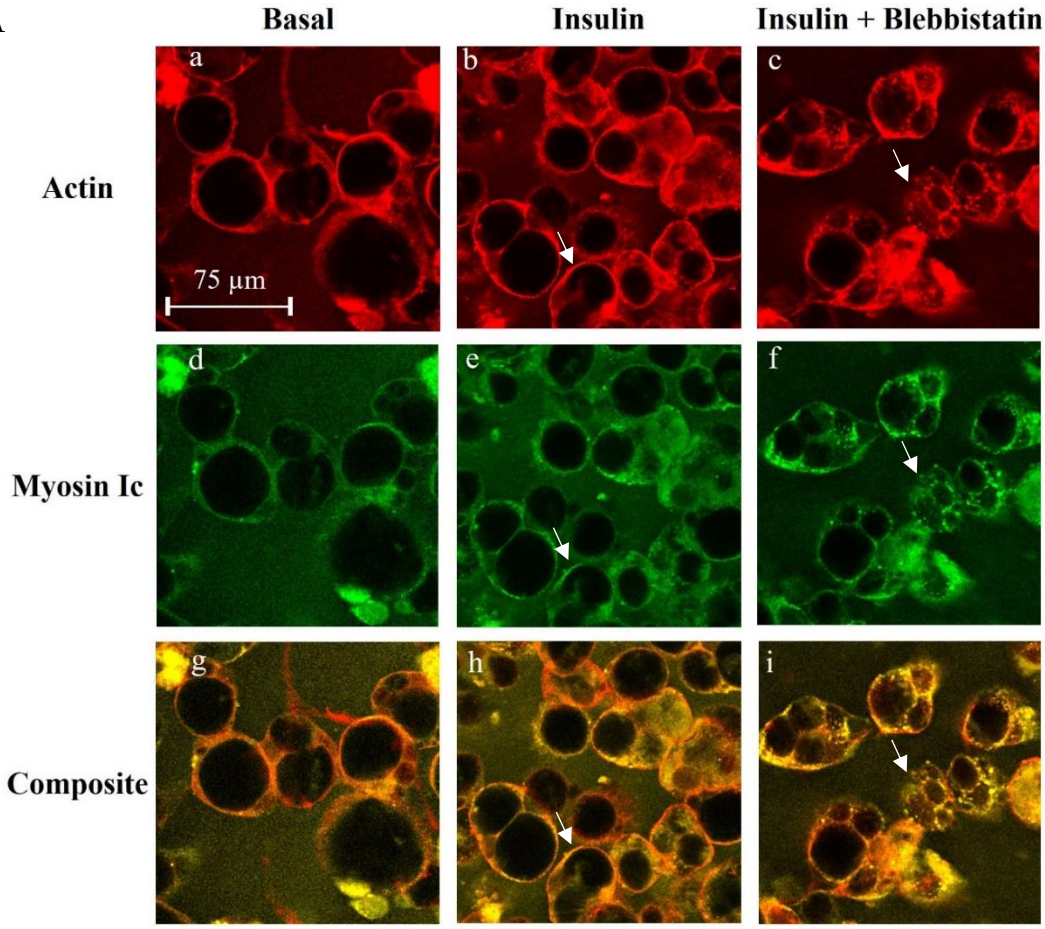
During insulin stimulation, F-actin is enriched at the plasma membrane and undergoes dynamic reorganizations in order to facilitate the trafficking of GLUT4 vesicles from intracellular pools to the plasma membrane in adipocytes[4]. It has been shown previously that if myosin II is inhibited during insulin stimulation, F-actin enrichment at the plasma membrane decreases which suggests that myosin II is necessary for actin filament enrichment[20]. During myosin II inhibition, it is possible that less actin filaments are available for myosin Ic and myosin Va to bind and use as secretory tracks for GLUT4 vesicles.

Thus, in order to determine if myosin II is required to maintain the actin tether on which myosin Ic and myosin Va promote GLUT4 translocation, we investigated the change in co-localization of myosin Ic and myosin Va with F-actin at the plasma membrane during insulin stimulation with and without myosin II inhibition.

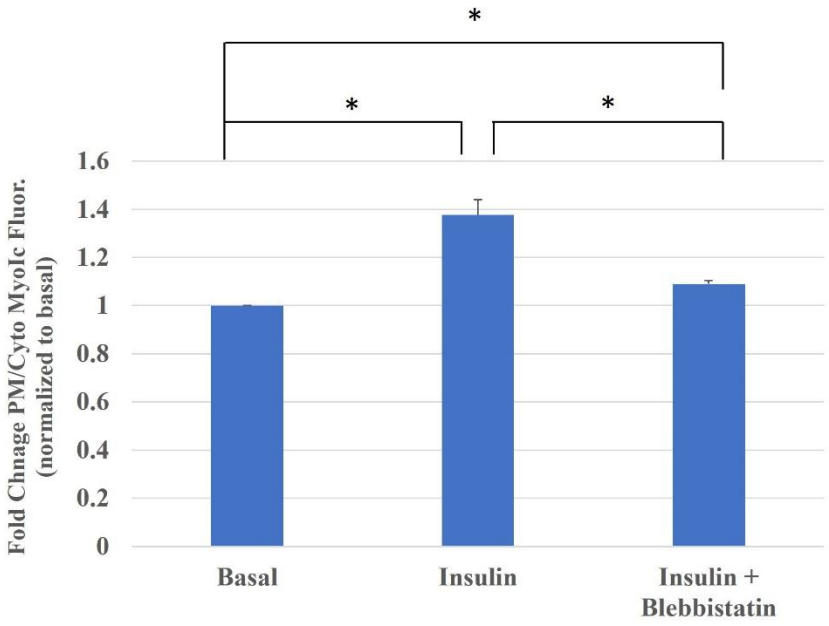
Inhibition of Myosin II Decreases Insulin Stimulated Co-localization of Myosin Ic with F-actin at the Plasma Membrane

In order to investigate if myosin II is necessary to co-localize cortical F-actin and myosin Ic, confocal microscopy was used to visualize myosin Ic and F-actin. F-actin was labeled using a Texas Red fluorophore conjugated to phalloidin, a toxin which preferentially binds F-actin by intercalating into the spaces between the globular actin monomers comprising actin filaments[35]. Mature adipocytes grown on coverslips were serum starved with 0.1% DMSO or 100 uM blebbistatin and subsequently treated with 100 nM insulin or left unstimulated, as described in the methods section.

A


Basal
Insulin
Insulin + Blebbistatin
Actin
Myosin Ic
Composite
a
b
c
d
e
f
g
h
i
75 µm

B


*
*
*
Fold Chnage PM/Cyto MyoIc Fluor. (normalized to basal)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Basal
Insulin
Insulin + Blebbistatin

C

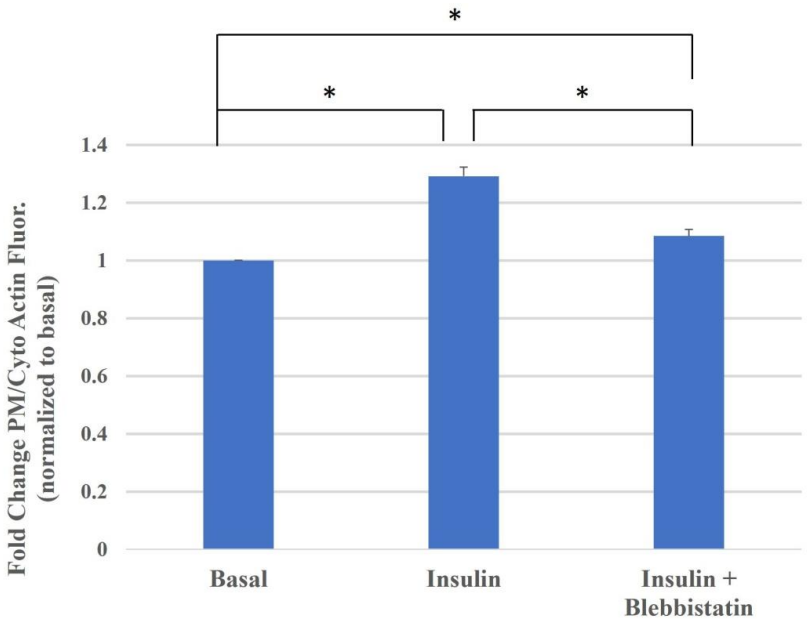


Figure 9. **A Decrease in Myosin Ic Localization at the Plasma Membrane is Observed During Insulin Stimulation and Myosin II Inhibition.** Adipocytes grown on coverslips were serum starved in the presence of vehicle (0.1% DMSO) or 100 uM Blebbistatin and subsequently treated with or without insulin. Coverslips were then fixed and permeabilized. Subsequently, adipocytes were incubated with primary antibody against myosin Ic. Appropriate secondary antibody was applied in addition to Texas Red-phalloidin to stain F-actin. **A)** Coverslips were then mounted and visualized using confocal microscopy. **B-C)** Plasma membrane and cytoplasmic fluorescence for myosin Ic and actin was measured using ImageJ. The ratio of plasma membrane to cytoplasmic fluorescence was normalized to the basal state. Each graph is expressed as the average of 3 independent experiments +/- SEM. *$p<0.05$.

Upon insulin stimulation there is an increase in fluorescent signal at the plasma membrane of myosin Ic and F-actin as observed with confocal microscopy (Figure 9A). Our results confirm previous studies indicating that insulin stimulation increases myosin Ic translocation to the plasma membrane[13,14]. Composite images for myosin Ic and F-actin fluorescence (Figure 9A. g, h, and i) indicate co-localization. Using ImageJ fluorescence quantification tools, we determined that actin filament enrichment at the

plasma membrane increased by approximately 30% (Figure 9C). These results are in agreement with previous studies in our lab which also observed an increase in F-actin at the plasma membrane during insulin stimulation. Similarly, myosin Ic enrichment at the plasma membrane also increased 37% at the plasma membrane (Figure 9B).

Upon co-treatment of adipocytes with insulin and blebbistatin, it was observed that myosin II inhibition reduced the insulin induced increase in F-actin formation at the plasma membrane (Figure 9C). Additionally, a decrease in myosin Ic fluorescence at the plasma membrane was also observed upon myosin II inhibition. Co-localization of myosin Ic and F-actin at the plasma membrane also appears to decrease under myosin II inhibition (Figure 9A. i). When compared to the basal state, F-actin enrichment at the plasma membrane only increased by 9% when treated with blebbistatin and insulin (Figure 9C). Similarly, myosin Ic translocation to the plasma increased by only 9% when treated with insulin and blebbistatin when compared to the basal state (Figure 9B). These results indicate that myosin II is necessary to maintain myosin Ic co-localization with F-actin at the plasma membrane.

Myosin II Inhibition Reduces Insulin-induced Myosin Va Co-localization with F-actin at the Plasma Membrane

To investigate if myosin II is necessary to localize myosin Va and F-actin at the plasma membrane, adipocytes were serum starved and treated using the same conditions as described in the previous section.

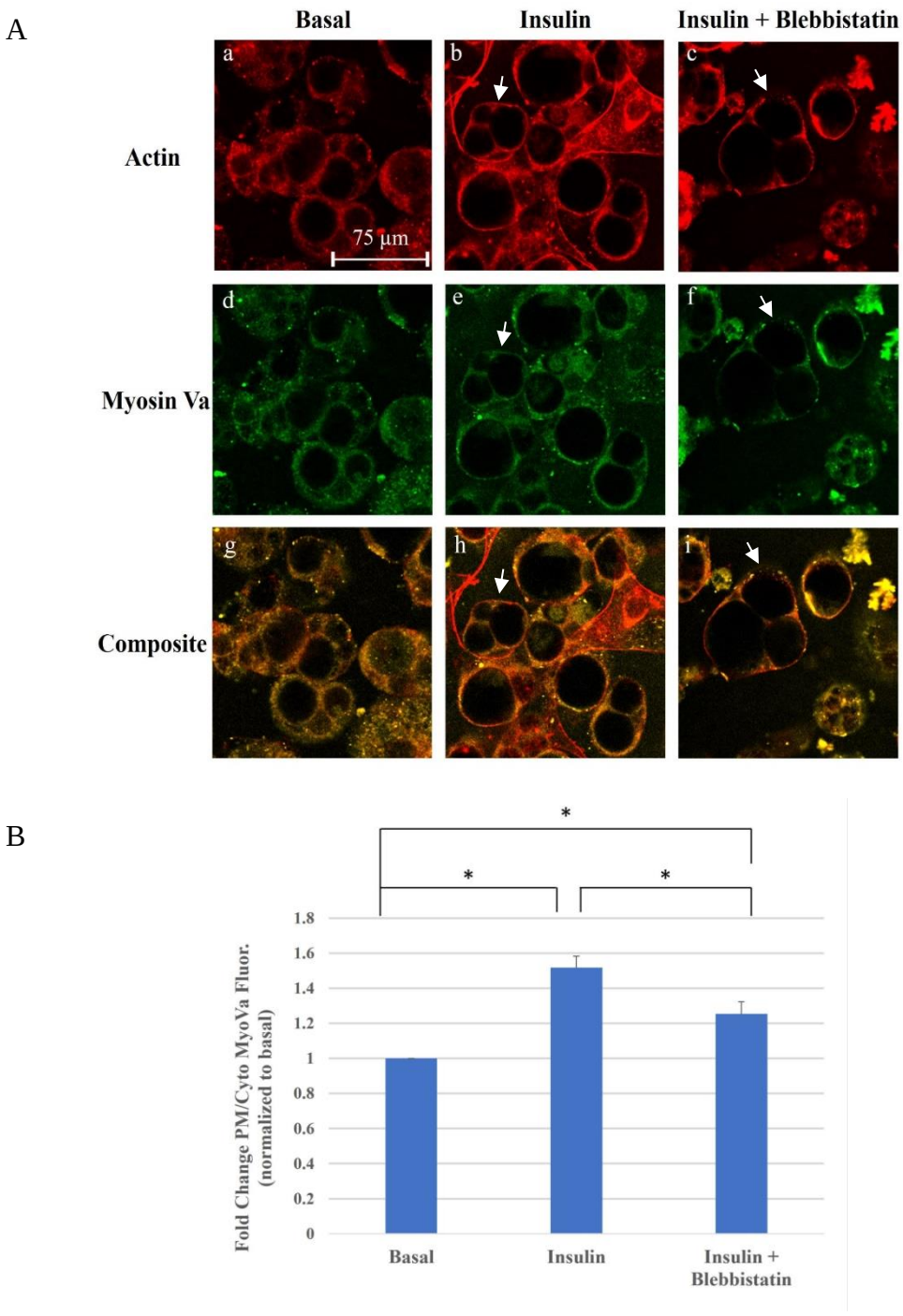


Figure 10. **During Myosin II Inhibition, a Decrease in Myosin Va Localization at the Plasma Membrane is Observed Upon Insulin Stimulation When Compared to Insulin Stimulation Alone.** Adipocytes grown on coverslips were serum starved in the presence of vehicle (0.1% DMSO) or 100 uM Blebbistatin and subsequently treated with insulin or without insulin. Coverslips were then fixed, permeabilized, and incubated with primary antibody against myosin Va followed by the appropriate secondary antibody. **A)**

Coverslips were then mounted and visualized using confocal microscopy. **B)** Plasma membrane and cytoplasmic fluorescence for myosin Va was measured using ImageJ. The ratio of plasma membrane to cytoplasmic fluorescence was graphed and normalized to the basal state. Each graph is expressed as the average of 3 independent experiments +/- SEM. *$p<0.05$.

In the basal state, myosin Va was observed to have primarily a diffuse, cytosolic localization; however, upon insulin stimulation an increase in myosin Va fluorescence at the plasma membrane was observed (Figure 10A). Our observations corroborate previous studies which also find increased levels of myosin Va at the plasma membrane upon insulin stimulation[12]. As seen previously, F-actin fluorescence at the plasma membrane also increased with insulin stimulation. When compared to the basal state, myosin Va localization at the plasma membrane increased by 51% (Figure 10B).

Upon myosin II inhibition with blebbistatin, myosin Va only increased by 25% at the plasma membrane when compared to the basal state. This is approximately 50% as much myosin Va translocation as was observed with insulin stimulation alone. These results indicate that, just as was seen with myosin Ic, myosin II is necessary to maintain co-localization of myosin Va with F-actin at the plasma membrane.

CHAPTER IV

DISCUSSION

While the cell signaling events prior to insulin-stimulated GLUT4 translocation have been well characterized, the cytoskeletal events prior to GLUT4 vesicle fusion with the plasma membrane, such as vesicle tethering, are less well characterized. Even less well known is the role myosin II plays during insulin-stimulated GLUT4 vesicle tethering. We sought to determine if myosin II stabilizes cortical actin tethers which support GLUT4 translocation to the plasma membrane during insulin stimulation.

In the present study, we demonstrated that myosin II inhibition prevents the localization of myosin Ic and GLUT4 at the plasma membrane upon insulin stimulation. Myosin Ic translocation to the plasma membrane was reduced upon myosin II inhibition from levels observed with insulin alone. GLUT4 also showed a decrease in translocation with blebbistatin treatment from insulin alone. This demonstrates that myosin II inhibition reduces the insulin-induced translocation of myosin Ic and GLULT4 suggesting that myosin II is indeed at least necessary to localize myosin Ic and GLUT4 at the plasma membrane. Next, we investigated if myosin II was necessary to co-localize myosin Ic and F-actin at the plasma membrane. Localization of myosin Ic and F-actin during insulin stimulation was observed using confocal microscopy. We observed that upon myosin II inhibition, myosin Ic and F-actin localization at the plasma membrane decreased from levels observed with insulin stimulation alone. These data show that

myosin Ic, GLUT4, and F-actin localize at the plasma membrane in a myosin II-dependent manner. Since myosin II has been shown previously to stabilizes actin filaments[31,36], these data suggest that myosin II is able to stabilize F-actin tethers at the plasma membrane, creating a stable platform to anchor myosin Ic and myosin Va to facilitate GLUT4 translocation to the plasma membrane.

Having established that these proteins localize at the plasma membrane together in a myosin II-dependent manner, we sought to determine if they also complex together in a myosin II-dependent manner. Immunoprecipitation of myosin IIa revealed that inhibition of myosin II decreases the ability of myosin Ic to complex with myosin IIa during insulin stimulation. Taken together these findings strongly indicate that myosin IIa is acting to stabilize the acto-myosin complex which is needed to promote GLUT4 exocytosis. It is important to note that blebbistatin reduces myosin II ATP-ase activity by complexing with myosin II, ADP, and Pi thereby slowing the release of the phosphate group after ATP hydrolysis[29]. During this time, the affinity of the myosin II head for actin is lower resulting in destabilized actin filaments.

Next, we also sought to assess if myosin Va localization and its ability to complex with other proteins, such as myosins II and Ic, and GLUT4, was affected by myosin II inhibition. Our results show that upon myosin II inhibition, myosin Va localization with GLUT4 at the plasma membrane decreased when compared to insulin treatment alone. Similarly, myosin Va localization with F-actin at the plasma membrane was decreased when myosin II was inhibited. These results suggest that myosin II is necessary to localize myosin Va, GLUT4, and F-actin at the plasma membrane. It was next necessary

to determine if there are any physical association of these proteins in a complex at the plasma membrane. Using immunoprecipitation of myosin Va, we showed that myosin IIa association with myosin Va during myosin II inhibition decreased below what is observed in the basal state. Myosin II inhibition also abolished the insulin-induced association of myosin IIa with myosin Va. These findings demonstrate that myosin IIa and myosin Va associate together in a myosin II-dependent manner.

Interestingly, during blebbistatin treatment, the association of myosin Ic and myosin Va with myosin IIa decreased below levels seen in the basal state. This suggests that, in the basal state, myosin Ic and Va may possibly associate at low levels with F-actin near the perinuclear region. Indeed, fluorescence microscopy images show enriched myosin Ic and myosin Va near the nucleus. F-actin as visualized by confocal microscopy is also found to be perinuclear in the basal state. This low level association of myosin Va and Ic with actin filaments is possible as myosin IIa is also localized near the perinuclear region where it may be associating with and stabilizing F-actin. Thus, one possible explanation for the observed decrease in association of myosin IIa with myosins Va and Ic below what is observed in the basal state is that myosin IIa would not be able to stabilize perinuclear actin filaments in addition to cortical actin filaments thereby reducing the amount of myosin Ic and myosin Va associating with myosin IIa below basal levels during myosin II inhibition.

Our findings corroborate previous evidence demonstrated by our lab which supports myosin II as an important regulatory protein promoting the process of GLUT4 exocytosis. Our lab has previously shown that upon myosin II inhibition in 3T3-L1

adipocytes, a variety of effects related to GLUT4 vesicle exocytosis and subsequent glucose uptake can be observed. First, glucose uptake is reduced with insulin stimulation[8]. As GLUT4 is the insulin responsive glucose transporter, a reduction in glucose uptake is likely due to a decrease in GLUT4 exocytosis which was observed during myosin II inhibition [28]. Although GLUT4 exocytosis is reduced in a state of myosin II inhibition, GLUT4 vesicles still approach the plasma membrane. This suggests that the GLUT4 vesicle is able to translocate only so far before an impediment, myosin II inhibition, prevents the final steps leading up to vesicle fusion and/or possibly prevents the fusion event itself. Our findings suggest that myosin II is promoting GLUT4 exocytosis by binding and stabilizing cortical actin tethers. Stabilized actin tethers provide a robust platform which are able to anchor myosin Ic and myosin Va aiding in GLUT4 translocation to the cell periphery. During myosin II inhibition, it is likely that actin tethers are compromised, resulting in potentially truncated tethers which bind myosin Ic and Va less effectively (Figure 4). This would lead to the observed reduction in GLUT4 exocytosis seen in our studies during myosin II inhibition using blebbistatin. Moreover, a similar reduction in GLUT4 exocytosis has also been observed in a study by Chung et al in which 3T3-L1 adipocytes were transfected with anti-myosin IIa siRNA[37].

Considering non-muscle myosin II's role in the cell as a critical structural and motor protein associated with the cytoskeleton, it is not surprising that myosin II inhibition would have an effect on GLUT4 exocytosis as it has been shown previously that a dynamic cytoskeleton is necessary for proper glucose uptake. Upon insulin stimulation both F-actin and myosin IIa are enriched at the cell cortex suggesting that

myosin IIa may be facilitating changes in the actin cytoskeleton which aid in GLUT4 exocytosis [4,20]. This case is further supported by Fulcher et al who demonstrated that myosin IIa is recruited to the plasma membrane specifically during insulin stimulation and co-localized with GLUT4 at the plasma membrane [28]. Moreover, it has been demonstrated that myosin II inhibition decreases the level of insulin-induced F-actin enrichment at the plasma membrane [20]. In the present study, we also show a reduction in the localization of myosin Ic and myosin Va with F-actin at the plasma membrane during myosin II inhibition. Together, these data provide further evidence that myosin II may be responsible for stabilizing actin tethers since myosin Ic and myosin Va cannot localize with F-actin at the plasma membrane when myosin II is inhibited.

Having provided evidence that myosin II is necessary to localize and associate myosin Ic and myosin Va with both GLUT4 and F-actin at the plasma membrane during insulin stimulation, future studies could investigate the localization patterns of myosin Ic and myosin Va using more specific methods to inhibit myosin II activity. As discussed earlier, myosin II assembles into bipolar filaments. These bipolar filaments bind F-actin and facilitate cytoskeletal rearrangements or cross-link F-actin to maintain cytoskeletal stability. By expressing myosin II proteins which are assembly incompetent, these experiments could be repeated to corroborate the current findings. Additionally, assembly incompetent constructs of each isoform of the myosin II heavy chain present in 3T3-L1s (myosin IIa and myosin IIb) could be used to determine which isoform contributes to the stabilization of actin tethers. Plasmids also exist which can over express non-phosphorylatable amino acid substitutions in the RLC [38]. Without RLC phosphorylation,

myosin II remains in its 10S hairpin conformation preventing myosin II from assuming its linearized, assembly competent form [39]. Thus, less F-actin stabilization would occur.

Together, our results give insight into the role of myosin II in supporting GLUT4 exocytosis. We have determined that myosin II inhibition decreases localization of myosin Ic and myosin Va with GLUT4 and F-actin at the plasma membrane. Additionally, we have determined that myosin II association with myosin Ic and myosin Va decreases with myosin II inhibition. These data suggest that myosin II is necessary to localize these proteins in an insulin-induced acto-myosin protein complex which supports GLUT4 translocation to the plasma membrane. These findings are important in further elucidating the distal events in insulin signaling. These events may be critical regulatory nodes which could yield therapeutic targets helpful in treating those with type II diabetes.

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